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Award Number: W81XWH-05-1-0135

TITLE: Using Genetically Engineered Mice to Probe the Role of Bioactive Lipids in Prostate Carcinogenesis

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REPORT DATE: July 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-07-2006	Final	15 Dec 2004 – 14 Jun 2006

4. TITLE AND SUBTITLE

Using Genetically Engineered Mice to Probe the Role of Bioactive Lipids in Prostate Carcinogenesis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0135

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Diana M. Stafforini

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Utah
Salt Lake City UT 84112-5550

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Prostate cancer (PCa) is the most common malignancy in North American men and represents the second most common cause of cancer death. PCa is characterized by many different stages including very aggressive forms that disseminates to bone, lymph nodes, and other tissues. Tumors release factors that attract and activate cells of the immune system including macrophages. In some tumors, macrophages can both stimulate and inhibit cancer growth and proliferation. One of the changes that take place when macrophages are exposed to specific stimuli is the transcriptional activation of a gene encoding a phospholipase A2, platelet-activating factor acetylhydrolase (PAF-AH) that has anti-inflammatory properties owing to its ability to hydrolyze a large group of bioactive lipids. The levels of PAF-AH are dramatically increased in PCa compared to normal prostate tissues; the source is likely macrophages recruited to PCa sites. We are testing the hypothesis is that elevated PAF-AH derived from macrophages recruited to PCa sites alters the rate of PCa progression using both in vivo and in vitro methodologies. We have successfully generated PAF-AH-deficient mice in a model of PCa (the TRAMP transgenic model) that recapitulates many salient aspects of human PCa. We are analyzing how deficiency of PAF-AH modulates PCa progression in vivo. In addition, we have used cellular approaches to establish that members of the PAF signaling axis promote growth of PCa cells and that overexpression of PAF-AH decreases cellular proliferation either by inhibiting cell division or by promoting apoptosis.

15. SUBJECT TERMS
BIOACTIVE LIPIDS ● PLATELET-ACTIVATING FACTOR ● KNOCKOUT MOUSE ● METASTASIS ● ANGIOGENESIS ● SURVIVAL ● VASCULARIZATION ● INFLAMMATION ● OXIDANTS ● OXIDIZED PHOSPHOLIPIDS ● PHOSPHOLIPASE A2 ● TUMOR GROWTH

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	9	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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INTRODUCTION

Prostate cancer (PCa) is the most common malignancy in North American men and represents the second most common cause of cancer death. PCa is characterized by many different stages including very aggressive forms that disseminate to bone, lymph nodes, and other tissues. The host's immune system plays a central role in the ability of tumors to obtain nutrients and oxygen. Tumors release factors that attract and activate cells of the immune system including macrophages. Macrophages have well-defined roles as agents that participate in inflammation and that help us fight infections, but the biological significance of tumor-associated macrophages (TAMs) is unclear (Elgert et al., 1998). When macrophages are recruited to tumor sites complex cancer-macrophage interactions take place (Joseph and Isaacs, 1998; Satoh et al., 2003). The biological result of these interactions depends on the changes that occur within macrophages, the physical location reached by these cells as they encounter the tumor, and the type of malignancy. In some tumors, including PCa, macrophages can both stimulate and inhibit cancer growth and proliferation (Shimura et al., 2000). One of the phenotypic changes that take place when macrophages are exposed to specific stimuli is the transcriptional activation of a gene encoding a phospholipase A_2, platelet-activating factor acetylhydrolase (PAF-AH) (Stafforini et al., 1990). This enzyme has anti-inflammatory properties owing to its ability to hydrolyze a large group of bioactive lipids including platelet-activating factor (PAF), oxidized phospholipids and esterified isoprostanes with varied and potent biological functions (Stafforini et al., 2003; Stafforini et al., 2006). The levels of PAF-AH mRNA, which provides a mechanism to down-regulate bioactive lipid signaling, are altered during PCa progression compared to normal prostate tissues. The PAF-AH mRNA detected in these analyses likely originates from macrophages recruited to PCa sites. *Our hypothesis is that elevated PAF-AH derived from macrophages recruited to PCa sites alters the rate of PCa progression.* We have developed genetically-engineered mice that lack the ability to inactivate the group of bioactive lipids mentioned above owing to a deficiency in the inactivating enzyme PAF-AH. Our approach to investigate how the PAF signaling pathway participates in PCa is based on the utilization of one of the best-characterized models of the disease (the TRAMP-transgenic model) that recapitulates many salient aspects of human PCa and that has been utilized for a wide range of studies, such as analyses of aberrant growth factor signaling in PCa progression and chemoprevention studies (Gingrich et al., 1996; Greenberg, 2000). To investigate the role of PAF-AH in PCa we proposed to test how deficiency of PAF-AH modulates cancer progression in the TRAMP model. The overall objective and scope of this research is to assess if, and how, PCa is modulated when the PAF/oxidized phospholipid signaling pathway is exacerbated by inhibition of PAF degradation.

BODY

The initial task we proposed to accomplish is listed below:

Task 1. Generate PAF-AH deficient animals in the transgenic adenocarcinoma mouse prostate (TRAMP) model

Task 1a (completed). Obtain a pair of male TRAMP-transgenic mice from commercially available sources (Jackson Laboratories) and set up genotyping analyses according to the distributor's recommendations.
Please see annual report covering December 2004-November 2005 funding period.

Task 1 b (completed). Breed a pair of male TRAMP-transgenic mice to 8 PAF-AH$^{-/-}$ female mice and identify progeny that are TRAMP-transgenic:PAF-AH$^{+/-}$ (months 1-2.5). The average litter size is 8, and theoretically 25% of the progeny should have the desired genotype. Thus, the 8 females are expected to generate 16 mice (8 males and 8 females) with the desired genotype.
Please see annual report covering December 2004-November 2005 funding period.

Task 1 c (completed): Breed 8 TRAMP-transgenic:PAF-AH$^{+/-}$ to 8 PAF-AH$^{-/-}$ mice. Breed 8 TRAMP-transgenic:PAF-AH$^{+/-}$ to 8 C57BL/6 mice. Identify male progeny that are TRAMP-transgenic:PAF-AH$^{-/-}$ and TRAMP-transgenic:PAF-AH$^{+/+}$.
We performed these crosses, weaned the animals, genotyped them, and identified animals that inherited the TRAMP transgene. TRAMP-positive animals then were genotyped for the presence of the PAF-AH allele. The summary of our results to date, is shown in Table I.

Table I. ***Characteristics of TRAMP-transgenic animals generated for this project as of July 13, 2006.***

Mouse ID	DOB	PAF-AH genotype	Mouse ID	DOB	PAF-AH genotype	Mouse ID	DOB	PAF-AH genotype
F2-23	7/8/2005	Wild-type	F3-3	9/24/2005	Heterozygous	F4-1	12/1/2005	Homozygous deficient
F2-24	7/8/2005	Wild-type	F3-4	9/24/2005	Heterozygous	F4-3	12/1/2005	Homozygous deficient
F2-26	7/8/2005	Wild-type	F3-6	9/24/2005	Heterozygous	F4-10	12/4/2005	Homozygous deficient
F2-27	7/8/2005	Wild-type	F3-7	9/24/2005	Heterozygous	F5-1	2/4/2006	Homozygous deficient
F2-34	7/28/2005	Wild-type	F3-16	9/30/2005	Heterozygous	F5-2	2/4/2006	Homozygous deficient
F2-36	7/28/2005	Wild-type	F3-17	9/30/2005	Heterozygous			
F2-37	7/28/2005	Wild-type	F3-20	9/30/2005	Heterozygous			
F2-40	8/5/2005	Wild-type	F3-27	10/17/2005	Heterozygous			
F2-43	8/5/2005	Wild-type	F5-5	2/12/2006	Heterozygous			
F2-55	9/21/2005	Wild-type						
F2-67	9/22/2005	Wild-type						
F2-68	9/22/2006	Wild-type						
F3-87	1/11/2006	Wild-type						
F3-91	1/11/2006	Wild-type						
F3-97	1/15/2006	Wild-type						
F3-105	1/15/2006	Wild-type						
F5-6	2/12/2006	Wild-type						

Task 2. Investigate the role of PAF-AH deficiency on primary tumor incidence, magnitude, progression, and survival.

Expression of PAF-AH protein and activity — During the course of our breeding strategy we generated a number of animals that were positive for the TRAMP transgene and wild-type in PAF-AH. These mice allowed us to become familiar with the techniques of genitourinary tract dissection and identification of prostate tumors as this is a new area of investigation for our group. In addition, the acquisition of tissue samples permitted us to establish and validate a number of biochemical assays that we will need to use for the remainder of the project. The results of our initial studies confirmed expression of PAF-AH activity and protein in the mouse prostate but not in other organs of the male genitourinary tract (**Figure 1**, lanes 1-2). Second, we found that the enzymatic activity of PAF-AH correlated with the levels of protein expressed, suggesting that in TRAMP mice, these parameters can be used interchangeably. Third, the presence of the TRAMP transgene resulted in decreased expression of PAF-AH at late stages of disease progression (29 weeks) compared to a wild-type animal of similar age (26 weeks). A young animal that had not yet reached puberty and that was both TRAMP transgenic and heterozygous for PAF-AH displayed activity and protein levels similar to those observed in a much older PAF-AH wild-type/TRAMP transgenic animal. These data, while preliminary, suggest that the levels of PAF-AH expression are higher in early lesions compared to later stages of disease progression.

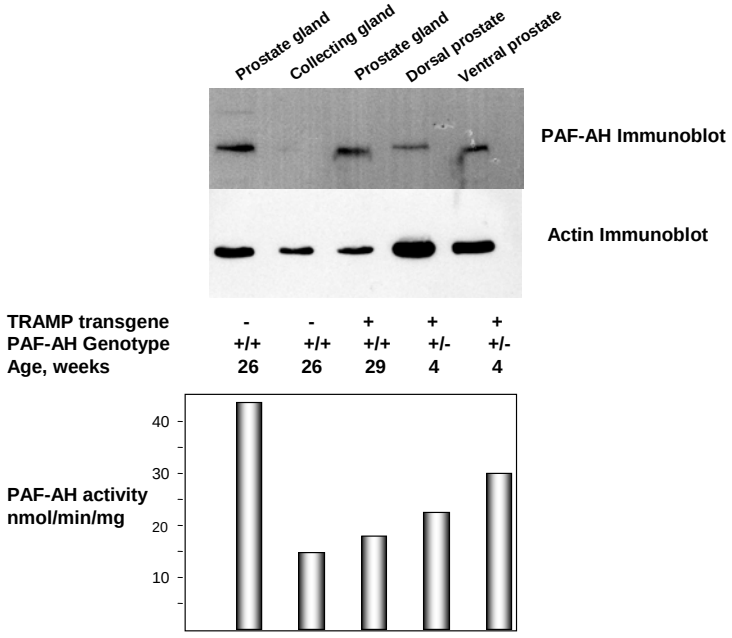

Figure 1. ***Expression of PAF-AH protein and activity in the prostate and collecting glands of wild-type and TRAMP-transgenic mice.***

The prostate and collecting glands of mice of the ages and genotypes indicated were harvested in Promega lysis buffer and 50-μg aliquots then were subjected to electrophoresis on SDS-PAGE gels. The proteins were transferred to PVDF membranes and probed with antibodies against PAF-AH (top panel) or actin (middle panel). The same samples were assayed for PAF-AH activity using a radiometric assay, as previously described (bottom panel) (Stafforini et al., 1990).

Effect of PAF-AH deficiency on the survival of TRAMP transgenic mice — During the course of our studies some of the mice generated perished unexpectedly and at random times. We analyzed the genotypes and life spans of these animals and found preliminary evidence for a role of PAF-AH deficiency in the life span of TRAMP transgenic mice (**Figure 2**). It is unlikely that this difference can be accounted by altered genetic backgrounds as these animals had similar mixed C57 BL6/FVB backgrounds. The data suggest that deletion of PAF-AH decreases the lifespan of TRAMP transgenic mice. In contrast, we found no differences in the life span of wild-type and PAF-AH deficient mice in the absence of the TRAMP transgene (not shown). These results suggest increase disease severity in animals that have decreased levels of expression of PAF-AH.

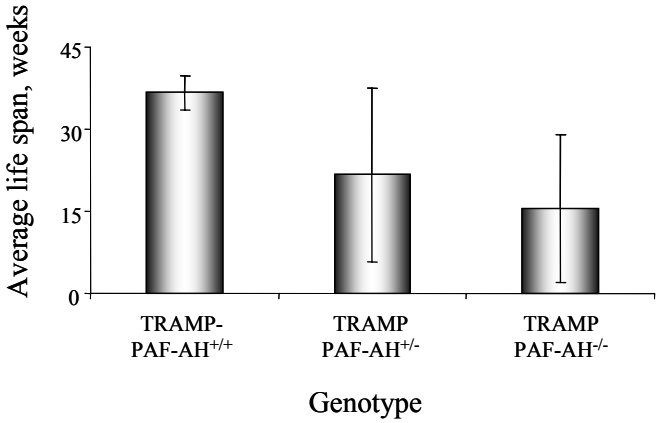

Figure 2. ***Deletion of PAF-AH decreases the life span of TRAMP transgenic mice.***

Each group consisted of three mice of the indicated genotypes. The animals perished naturally and the times of death were recorded and plotted.

Prostate tumors and metastatic disease — As our colonies developed we found that tumor formation was more evident in TRAMP transgenic animals that lacked PAF-AH compared to TRAMP transgenic:PAF-AH wild-type littermates. The most dramatic effect we observed is depicted in **Figure 3**. While these two animals differed in age and it is possible that the severity of the tumor observed in the PAF-AH deficient animal occurred owing to its age, we have not yet observed tumors of this magnitude or severity in any of our TRAMP PAF-AH wild-type animals, even at much older ages. In addition, we have observed metastases to the liver and head/neck areas in the studies conducted so far. The incidence of metastases was much lower in TRAMP transgenic mice that had normal levels of PAF-AH expression compared to animals that lacked normal expression of the enzyme.

Figure 3. ***Dissection of prostate glands and tumors in TRAMP transgenic mice.***

Illustration of two animals undergoing dissection of the genitourinary tract for further histological and biochemical analyses. The animal shown in the left panel was a 19.5 week-old TRAMP transgenic mouse. The animal on the right was a 30 week-old TRAMP transgenic mouse that lacked expression of PAF-AH.

Additional Task: Molecular and cellular studies.

In our previous Annual Report we presented evidence supporting a role for PAF in prostate cancer cell growth and we also provided results suggesting that PAF-AH attenuated the basal growth of LNCaP and PC-3 (not shown) prostate cancer cells in culture (see **Fig. 3** of our previous report). Our results suggested that PAF-AH functions to restore cellular homeostasis in the prostate epithelium by degrading substrates that either promote cellular growth or inhibit apoptosis. These data provided a useful model system to characterize the contribution of the bioactive lipid signaling pathway we are studying to the pathogenesis of PCa. In related experiments we investigated the levels of expression of PAF-AH in prostate cancer cells at various stages of differentiation. Based on the fact that our results in the TRAMP model suggested a role for PAF-AH deficiency as a severity factor, we reasoned that the state of cellular differentiation might correlate with the levels of PAF-AH expression. We utilized primary prostate

epithelial cells and three prostate cancer cell lines at various stages of differentiation and determined mRNA levels for both PAF-AH and GAPDH. We found (**Figure 4**) that prostate epithelial cells in culture expressed PAF-AH mRNA, that a highly differentiated prostate cancer cell line (LNCaP) had upregulated levels of expression and that PAF-AH expression was attenuated as the degree of differentiation decreased to that characteristic of PC-3 and DU-145 cells. These results were in agreement with a model wherein elevated PAF-AH levels occur as a response to pro-inflammatory and other stimuli, and that this response is blunted during later stages of disease progression.

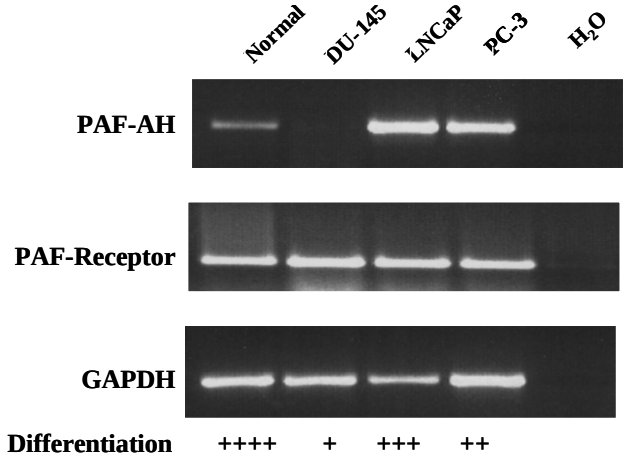

Figure 4. ***Expression of PAF-AH mRNA in human prostate epithelial cells and in prostate cancer cells at various stages of differentiation.***

The cells indicated in each case were maintained in culture and confluent layers were harvested. We isolated total RNA using TRIZOL reagent, according to the instructions provided by the manufacturer (Invitrogen). One μg of RNA was subjected to reverse transcription and the cDNA was subsequently amplified using primers specific for PAF-AH (top panel), the PAF receptor (middle panel) and GAPDH, as a control (lower panel). The levels of PAF receptor relative to GAPDH mRNA levels were similar in all the cell lines tested.

In additional studies we investigated whether differentiated prostate cancer cells had the ability to respond to PAF and related inflammatory stimuli by upregulating expression of PAF-AH. We treated LNCaP cells with various levels of PAF under conditions that prevent ligand degradation. We then measured PAF-AH mRNA and protein levels and found that LNCaP cells responded to low levels of PAF by upregulating PAF-AH expression (**Figure 5)**. However, this response was blunted at higher, yet physiologically relevant, ligand levels. These results again supported a model wherein the regulation of PAF-AH synthesis in response to engagement of the PAF receptor in prostate cancer cells is subject to tight regulatory control mechanisms. Decreased levels of PAF-AH resulting from over-stimulation or by genetic deletion (as in our animal model) may worsen disease outcome by increasing the rate of cellular proliferation through mechanisms that involve, at least in part, participation of the PAF receptor.

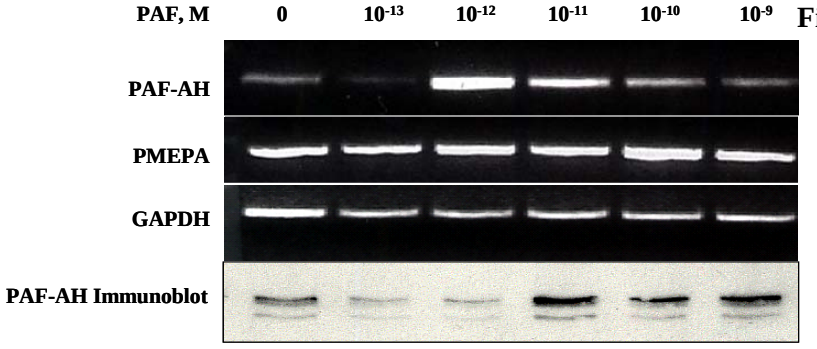

Figure 5. ***LNCaP cells respond to PAF stimulation by increasing the levels of PAF-AH mRNA.***

LNCaP cells were exposed to the indicated concentrations of PAF in the presence of serum pretreated with Pefabloc to inactivate the endogenous PAF-AH activity. The levels of PAF-AH mRNA and protein then were determined by RT-PCR and immunoblot analyses, respectively. GAPDH and PMEPA (an androgen-dependent gene product) are shown for comparison.

KEY RESEARCH ACCOMPLISHMENTS RELEVANT TO THIS REPORT

- Established dissection, biochemical and immunological assays necessary for the execution of the study
- Generated genetically-engineered animals with the genotypes required for the execution of the study
- Investigated expression of PAF-AH in the prostate gland of normal and TAMP transgenic animals with normal and decreased levels of PAF-AH
- Obtained evidence for modulation of PAF-AH expression in the prostate gland during development of prostate cancer
- Observed decreased life span of mice that lack expression of PAF-AH during prostate cancer

development.

- Initiated analysis of tumor formation within the context of PAF-AH deletion and generated initial data suggesting increased severity of tumor formation and metastasis induced by PAF-AH deficiency.
- Complemented animal studies with molecular analyses in prostate epithelial and prostate cancer cells.
- Observed altered levels of basal PAF-AH expression in prostate epithelial and prostate cancer cells at various stages of differentiation.
- Demonstrated the ability of differentiated prostate cancer cells to respond to PAF by upregulating PAF-AH mRNA and protein levels.

REPORTABLE OUTCOMES

Presentations: This work has been presented on several occasions in our institution where it is open for discussion and subject to input and criticism from colleagues, trainees, and students. Moreover, portions of this work were presented by the PI to scientists at the Oswaldo Cruz Foundation in Rio de Janeiro, Brazil.

Animal models: As a consequence of our work, a new line of mice has been generated that harbors the TRAMP transgene developed by Dr. Greenberg and his group and in which one or two PAF-AH alleles are deleted. These animals constitute excellent tools to probe the role of PAF-AH and the PAF signaling pathway in prostate carcinogenesis.

CONCLUSIONS

The results we have obtained so far using both cellular and *in vivo* studies are consistent with the hypothesis that the PAF signaling pathway contributes to the pathogenesis of prostate cancer by modulating cellular growth. Both lines of work suggest that phenotypic alterations that lead to elevated expression of PAF-AH and thus result in silencing of signals elicited by bioactive lipid mediators through the PAF receptor may inhibit growth and/or promote apoptosis. Conversely, in the absence of PAF-AH, the severity of the disease is higher with increased incidence of metastases and tumor growth. In the setting of prostate cancer, this phenotype is consistent with a model of recruitment and activation of macrophages by inflammatory stimuli, resulting in the localized release of a PAF-AH activity which may serve to blunt the actions of bioactive lipids such as PAF. This response seems to be blunted at later stages of differentiation. Our data continue to support out initial prediction that PAF-AH deficiency exacerbates PCa. The approaches we are utilizing are complementary in nature, include both human and murine systems, and utilize an animal model with proven resemblance to the human disease.

"So what" section: the experimental design of our studies is based on the need to define what types of patients would potentially benefit the most from this work. The use of the animal model proposed here ensures that various phases of PCa development are represented. This feature constitutes a powerful tool, because we will be able to identify the precise step affected by the PAF/oxidized phsopholipid signaling pathway and the enzyme that suppresses these signals. Thus, we are in a position to test if initiation, progression and/or resolution are affected. This information will help us define the subset of PCa patients who would benefit the most from potential targeted therapies in the future. There may also be diagnostic applications generated from the results of these studies. Because it is possible to measure the levels of PAF-AH in blood and presumably in prostate biopsies, we will be able to identify the levels of this protein in patients using clinical assays and genetic tests. This will allow us to investigate if altered functioning of this gene affects PCa and then use this information to predict risk. Thus, the clinical applications that could result from the work proposed here have both diagnostic (assessment of gene and protein status in PCa patients) and interventional (*i.,e*, administration of recombinant protein) applications. Finally, the relevance of the studies proposed here lies on the potential discovery that the PAF signaling pathway plays a role in PCa and that altered levels of the enzyme that silences this pathway may modulate progression and dissemination of the disease. If these findings are confirmed, they will further our understanding of one the mechanisms that govern PCa. Therapies aimed at inhibiting PCa growth or spreading by targeting this pathway alone or in combination with others, could be developed. For example, the use of gene-modified macrophage therapy treatment using intratumor injection of PAF-AH-transduced macrophages could be considered as a complement to existing therapies using IL-12-transduced macrophages (Satoh et al., 2003). Finally, new potential targets for intervention within this lipid-signaling pathway can be identified. For example, inhibition of the synthetic enzymes involved in PAF biosynthesis would offer an additional attractive possible target for intervention. And, inhibition of lipid oxidative reactions that can generate ligands for the PAF receptor such as oxidized phospholipids, could also be contemplated as a potential interventional approach.

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APPENDICES

NOT APPLICABLE